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Molecular interaction in the mouse PAG between NMDA and opioid receptors in morphine-induced acute thermal nociception

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Abstract

Previous evidence demonstrates that low dose morphine systemic administration induces acute thermal hyperalgesia in normal mice through μOR stimulation of the inositol signaling pathway. We investigated the site of action of morphine and the mechanism of action of μOR activation by morphine to NMDA receptor as it relates to acute thermal hyperalgesia. Our experiments show that acute thermal hyperalgesia is blocked in periaqueductal gray with the μOR antagonist CTOP, the NMDA antagonist MK801 and the protein kinase C inhibitor chelerythrine. Therefore, a site of action of systemically administered morphine low dose on acute thermal hyperalgesic response appears to be located at the periaqueductal gray. At this supraspinal site, μOR stimulation by systemically morphine low dose administration leads to an increased phosphorylation of specific subunit of NMDA receptor. Our experiments show that the phosphorylation of subunit 1 of NMDA receptor parallels the acute thermal hyperalgesia suggesting a role for this subunit in morphine-induced hyperalgesia. Protein kinase C appears to be the key element that links μOR activation by morphine administration to mice with the recruitment of the NMDA/glutamatergic system involved in the thermal hyperalgesic response.

Keywords: hyperalgesia, morphine, mu opioid receptor, NMDA, phosphorylation, protein kinase C.
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Systemic administration of morphine at low doses has been shown to elicit acute hyperalgesia in animal models of pain, i.e., tail flick, Freund's adjuvant-induced arthritic rats and hot plate (Kayan *et al.* 1971; Kayser *et al.* 1987; Crain and Shen 2001; Galeotti *et al.* 2006). A specific signaling pathway for morphine-induced acute thermal hyperalgesia was previously identified in mouse via mu opioid receptor (μOR) activation of the phospholipase C beta 3 isoenzyme (PLCβ_3) and protein kinase C gamma (PKCγ) nociceptive pathway (Galeotti *et al.* 2006). *N*-methyl-D-aspartate (NMDA) receptor activation was also shown to play a role in low dose morphine-induced acute hyperalgesia suggesting that glutamatergic system is implicated in the excitatory response elicited by low dose opioid administration (Holtman and Wala 2005; Galeotti *et al.* 2006). However, the site of action of morphine and the mechanism of action of μOR activation by morphine to NMDA receptor are presently unknown as they relate to acute thermal hyperalgesia. Experiments performed in cultured neurons demonstrate that μOR opioids facilitate the activation of NMDA receptors in neurons of brain regions through activation of protein kinase C (PKC) (Chen and Huang 1991; Martin *et al.* 2001). This enzyme associates physically with NMDA receptor 1 (NR1) subunit in the post-synaptic density in rat brain, leading to enhancement of synaptic activity (Suen *et al.* 1998). Post-translational modification including phosphorylation modulates the function of NMDA receptors which are phosphorylated on serines of both NR1 and NR2 subunits and on tyrosine of the NR2 subunit (Sanchez-Perez and Felipo 2005). Morphological evidence showed that the majority of NR1 labeled dendrites contained μOR labeling in periaqueductal gray (PAG) (Commons *et al.* 1999). This region has been

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Abbreviations used: NMDA, *N*-methyl-D-aspartate; NR1, NMDA receptor1; PAG, periaqueductal gray; PKC, protein kinase C; S890, serine 890.; μOR, mu opioid receptor.

identified as a key component of the pain inhibitory system since electrical or chemical stimulation of this area suppresses nociceptive transmission from the dorsal horn of the spinal cord (Basbaum and Fields 1984). Importantly, it has been suggested that opioid effect may extend beyond inhibition in the PAG. In brain slices, the excitatory action of NMDA on PAG neurons is potentiated by a μOR agonist at low nanomolar concentration (Kow *et al.* 2002). These results support the hypothesis that the site and the mechanism of action of morphine low dose, as they relate to acute thermal hyperalgesia, might reside in PAG. Therefore, we choose to investigate whether μOR and NMDA antagonist administration to PAG reverts the thermal hyperalgesic effect induced by low dose morphine systemic acute administration to mice. The effect of morphine on phosphorylation of the NMDA receptors in PAG are studied as it relates to acute thermal hyperalgesic effect.

Materials and methods

Animals

Sexually mature male Swiss Webster mice (Morini, S. Polo d'Enza, Italy), weighing 23–30 g, were used. The animals were fed a standard laboratory diet and water *ad libitum*, kept at 23 ± 1°C with a 12-h light/dark cycle and previously habituated to the laboratory according to Abbot (Abbott *et al.* 1986). All experiments were carried out in accordance with the European Community Council Directive of November 24 1986 for the care and use of laboratory animals.

Drugs

The following drugs were used: D-Phe–Cys–Tyr–D-Trp–Orn–Thr–Pen–Thr–NH_2 (CTOP), MK801, chelerythrine and morphine HCl (Sigma Chemicals St Louis, MO, USA). Drugs were administered in a volume of 0.2 μL per mouse by intracranial infusion, and 10 mL/kg by subcutaneous (s.c.) injection. All drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Morphine was administered s.c.; CTOP, chelerythrine and MK801 were administered intra-PAG immediately before morphine administration; saline was administered both s.c. and intra-PAG.

Surgery and microinjection

Cannula implantation was performed according to the procedure by Carvalho-Netto (Carvalho-Netto *et al.* 2007). Mice were implanted with 7-mm stainless steel guide cannula (26-gauge) under anesthesia. Stereotaxic coordinates (Paxinos and Franklin 2001) for the PAG were, respectively, 4.1 mm posterior to bregma, 1.3 mm lateral to the midline, and 2.2 mm ventral to the skull surface, with the guide cannula angled 26° to the vertical. A dummy cannula (33-gauge stainless steel wire) was inserted into each guide-cannula immediately after each surgery to reduce the incidence of occlusion. Five to seven days after surgical recovery, solutions were injected into the PAG by microinjection unit which extended 1.0 mm beyond the tips of the guide cannula. Each microinjection unit was attached to a 5-μL Hamilton microsyringe via polyethylene tubing and administration was controlled by an infusion pump programmed to deliver a volume at rate of 0.1 μL over a period of 30 s.

Hot plate test

The adopted method was previously described (O'Callaghan and Holtzman 1975). Mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1°C in a precision water-bath. We used a lower temperature in the hot plate test (52.5°C instead of 54°C) to reveal potential, subtle alterations that may occur in basal thermal nociception. Reaction times (s) were measured with a stopwatch immediately before morphine or vehicle administration and 15, 30, and 45 min after; each treatment was blind to the experimenter. The endpoint for the licking response was the first paw lick of the front paw. Those mice scoring < 12 and more than 18 s in the pre-test were rejected. All treatments for a same experiment were performed on the same period of time. Hot plate test for assaying the effect of different treatments on morphine hyperalgesia were performed on the same time respectively for CTOP or MK801 or chelerythrine administration to mice. All experiments were performed at the same hours (9–13 AM). Mice were randomly assigned to each group. Dose–response experiments were done using independent groups.

Effect of treatments on spontaneous motility, inspection activity and motor coordination

Groups of mice treated with all the used drugs at the highest doses and saline administered as described in the above experiments, were submitted to rota-rod (Vaught *et al.* 1985) and hole-board (Ghelardini *et al.* 2002) behavioral tests. The apparatus of rota-rod test consists of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotation speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. Performance time was measured before and 15, 30, and 45 min after administration of the investigated compounds. The hole board test consisted of a 40 cm square plane with 16 flush-mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. Twelve mice per group were tested. The skin temperature of the mice paws was measured by infrared thermometer (152-IRB thermometer, 2B, Varese, Italy) before and after administration of the different treatments or saline.

Western blot

Mice were administered with morphine (1 μg/kg), or saline in the absence or the presence of drugs. Mice were killed at 15, 30, 45 min and 60 min after morphine or saline administration. Brain was removed and put immediately in liquid nitrogen. Midbrain was blocked out and PAG samples were isolated under stereomicroscope

control (MZ12.5 Leica, Leica, Wetzlar, Germany). Western blot analysis was performed as previously described in detail (Pan *et al.* 1995). In summary, membrane homogenates (1 μg/μL protein) from PAG brain regions of pre-treated or untreated mice were solubilized in sodium dodecyl sulfate buffer and separated on 10% polyacrylamide gels. PAG samples pooled from three brain mice were used throughout the experiments. Proteins were transferred to nitrocellulose (1.5 h at 190 mA) and the membranes were blocked in phosphate-buffered saline containing 3% BSA for 1 h before addition of primary antisera. NMDA NR1 (Upstate, Lake Placid, NY, USA) and Sc-5274 (β-tubulin) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as probes at 1 : 1000 dilution. The same procedure as above was followed to detect NR1 subunit only when phosphorylated at serine 890 (Cell signalling, Beverly, MA, USA) or serine 896 (Upstate) except that: blotted nitrocellulose membranes were blocked for 1 h in freshly prepared 1% BSA-0.1% Triton X100-TBS (50 mmol/L Tris, 150 mmol/L NaCl pH 7.4) (T-TBS) and incubated for 4 h in 2 mmol/L $MnCl_2$, 1% BSA, 0.1% Triton X-100-TBS; T-TBS was used for following incubations. The specificity for phosphorylated protein of both the above polyclonal antibodies was previously tested (Brenner *et al.* 2004; Sanchez-Perez and Felipo 2005). To further test the specificity of antibodies, a western blot was cut between two lanes, stripped and one side was treated with lambda protein phosphatase (Upstate) and the other side with dilution buffer; the blot was reprobed with anti-NR1 serine 890 or 896 antibodies. All the blottings were visualized using a chemiluminescence detection system (Super Signal West Fento, Pierce Biotechnology Inc., Rockford, IL, USA) and quantified with the Versa Doc 1000 Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Protein was determined by the Lowry method (Lowry *et al.* 1951) using BSA as standard. PAG samples from each respective treatment group were processed on the same gel. Tubulin was used to verify uniform protein loading. The differences observed as density average values corresponding to saline and treated mice were expressed as a percentage of saline values. The relationship between morphine dose and the magnitude of the phosphorylation density values obtained in PAG sample at 15 min after morphine administration, was fitted to a dose-effect curve according to the model:

$$\text{Intensity of effect} = \frac{E_{\max}.C}{EC_{50}+C} \quad (1)$$

where C is the concentration of the drug, $E_{\max}$ is the maximum effect and EC_{50} is the dose which corresponds to 50% of the maximum effect. The experimental data will be fitted to the dose–effect mathematical model previously described using Inplot program (GraphPad InPlot, San Diego CA, USA).

Histology

At the conclusion of the experiments 1% Evans blue dye was administered to mice according to the microinjection procedure described above for intra-PAG administration. A post-mortem histological control of the injection site was performed on cryostat sections of unfixed brains previously frozen as described for the western blot methods. The data of any mice were excluded from statistical analysis if the cannula tip was outside the PAG or if the region had sustained extensive damage. The brains from mice which were further submitted to western blot were extracted leaving the cannula implanted in the brain. The location of cannula inside PAG was observed under stereomicroscope (Leica MZ12.5, Leica, Solms, Germany). Only specimens from mice with cannula path inside the PAG were used for western blot experiments.

Statistical analysis

All experimental results were given as the mean ± SEM. Analysis of variance ANOVA, followed by Fisher's protected least significant difference procedure for *post hoc* comparison were used to verify significance between two means of behavioral or immunoblotting density results. Data were analyzed with the StatView software for the Macintosh (1992). A significance level (α) < 0.01 was considered significant.

Results

Effect on hyperalgesia of μOR receptor and NMDA antagonist

The thermal hypernociception induced by morphine was prevented by pre-treatment with CTOP at 80 ng (Fig. 1). Lower doses were ineffective. Baseline level were re-established for all pre-treatments at 45 min after morphine administration. CTOP, when administered alone, did not modify the licking latency of mice in comparison with saline administered animals (Fig. 1). The administration of site-specific NMDA receptor antagonist MK801 to PAG produced a dose-dependent inhibition of the hyperalgesic effect induced by 1 μg/kg s.c. morphine administration to mice

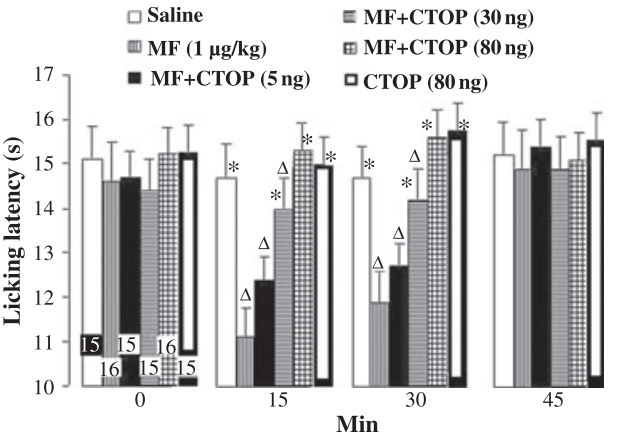

Fig. 1 Prevention of morphine induced hyperalgesia by PAG pre-treatment with CTOP at different doses. The licking latency values represented in figure were measured before (0), 15, 30, and 45 min after s.c. morphine administration (1 μg/kg) in presence or absence of CTOP infused to PAG at increasing doses. Vertical bars represent SEM; *and Δ = α < 0.01 significant difference in comparison respectively with 1 μg/kg morphine or s.c. saline induced licking latency value measured at the same time. Each value represents the mean ± SEM of the licking latency values. The number of mice used for each treatment is reported on vertical bars at 0 time. MF = morphine.

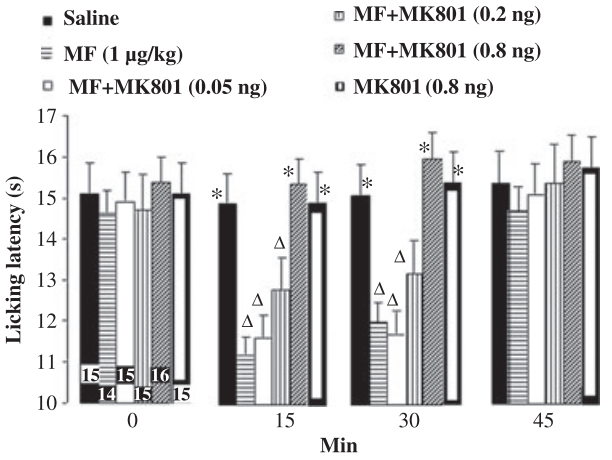

Fig. 2 Prevention of morphine-induced hyperalgesia by PAG pre-treatment with MK801 at different doses- The licking latency values represented in figure were measured before (0), 15, 30, and 45 min after intra-PAG MK801 infusion or s.c. morphine administration (1 μg/kg) in presence or absence of MK801 at increasing doses. Vertical bars represent SEM; * and Δ = α < 0.01 significant difference in comparison respectively with 1 μg/kg morphine or s.c. saline induced licking latency value measured at the same time. Each value represents the mean ± SEM of the licking latency values. The number of mice used for each treatment is reported on vertical bars at 0 time. MF = morphine.

(Fig. 2). The treatment with MK801 at 0.8 ng completely reversed to basal value morphine-induced hyperalgesia. MK801, when administered alone, did not modify the

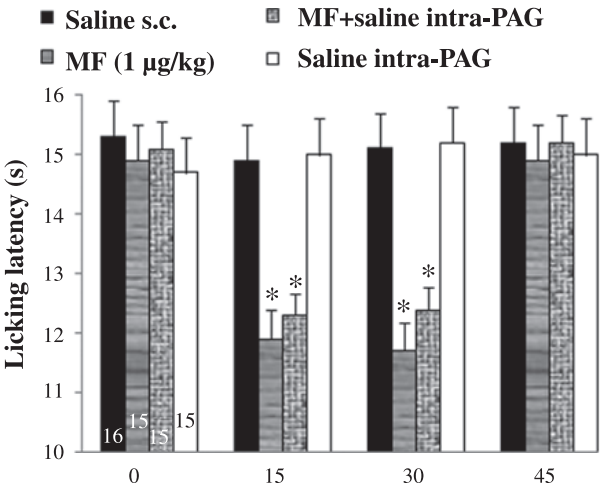

Fig. 3 Morphine hyperalgesic effect after intra-PAG saline administration- The licking latency values represented in figure were measured before (0), 15, 30, and 45 min after s.c. morphine administration (1 μg/kg) in presence or absence of intra-PAG saline administration. Vertical bars represent SEM value measured at the same time. Each value represents the mean + SEM of the licking latency values; * = α < 0.01 significant difference in comparison with s.c. saline induced licking latency value measured at the same time. The number of mice used for each treatment is reported on vertical bars at 0 time. MF = morphine.

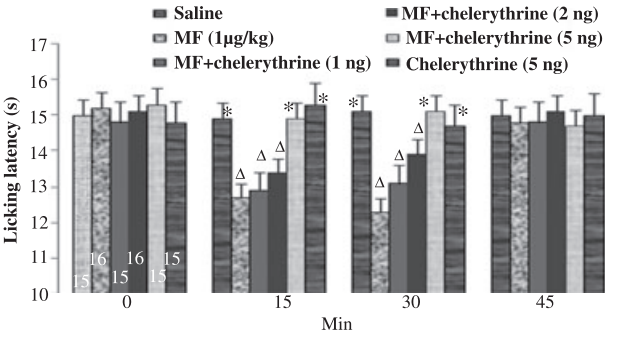

Fig. 4 Prevention of morphine-induced hyperalgesia by PAG pre-treatment with chelerythrine at different doses. The licking latency values represented in figure were measured before 0, 15, 30, and 45 min after intra-PAG chelerythrine infusion or s.c. morphine administration (1 μg/kg) in presence or absence of chelerythrine at increasing doses. Vertical bars represent SEM; * and Δ = α < 0.01 significant difference in comparison respectively with 1 μg/kg morphine or saline induced licking latency value measured at the same time. Each value represents the mean + SEM of the licking latency values. The number of mice used for each treatment is reported on vertical bars at 0 time. MF = 1 μg/kg morphine.

licking latency of mice in comparison with saline administered animals (Fig. 2). Baseline level were re-established for all pre-treatments at 45 min after morphine administration. Thermal hyperalgesic effect elicited by 1 μg/kg s.c. morphine administration to mice was unmodified by intra-PAG saline injection pre-treatment (Fig. 3).

Effect of PKC inhibitor on hyperalgesia

The PKC inhibitor chelerythrine, administered to PAG at different doses, completely reversed the 1 μg/kg morphine hyperalgesic effect at the higher dose (Fig. 4). A return to basal level was observed at 45 min after morphine administration for all chelerythrine doses. Chelerythrine, when administered alone, did not modify the licking latency of mice in comparison with saline administered animals (Fig. 4).

Effect of treatments on mouse motor coordination and spontaneous motility

Mice pre-treated with s.c. morphine, intra-PAG CTOP, MK801 and chelerythrine at the highest doses used in experiments previously described, were evaluated for motor coordination by use of the rotarod test and for spontaneous motility and inspection activity by use of the hole board test. The spontaneous motility as well as inspection activity of mice were unmodified by treatment with drugs in comparison with saline administered group (Fig. 5a). The number of falls from the rotating road evaluated before and 15, 30, and 45 min after the beginning of the rotarod test, showed the lack of any impairment in motor coordination of mice pre-treated with the above drugs in comparison with saline group

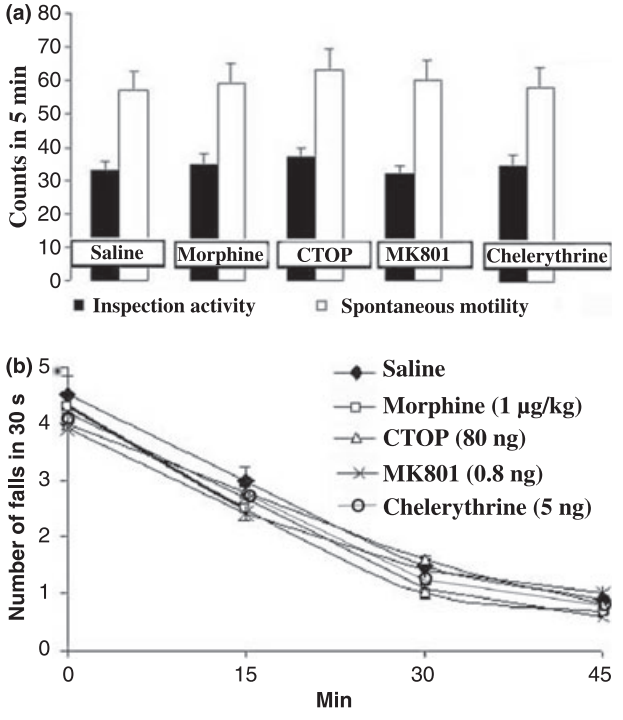

Fig. 5 Lack of effect by different treatment morphine (1 μg/kg), CTOP (80 ng), MK801 (0.8 ng) and chelerythrine (5 ng) administered to mice at higher effective doses on spontaneous motility/inspection activity (a) and motor coordination (b). Number of falls in 30 s measured in treated mice was compared with s.c. saline values measured at the same time (b). Vertical lines represent SEM.

(Fig. 5b). These behavioral tests show that drugs used in hot plate test experiment do not induce any significant change in motor coordination and spontaneous motility. The skin temperature of the mice paws remained unchanged after administration of the different pre-treatments or after surgery (data not shown) showing that different treatments or surgery do not induce any significant change.

Immunoblotting

Immunoblotting analysis revealed a significant increase of S890 NR1 phosphorylation with respect to basal value in PAG after 1 μg/kg morphine s.c. administration at 15, 30, and 45 min; 60 min after morphine administration the density value was not significantly different (Fig. 6a and b). NR1 content was not significantly modified by morphine administration (Fig. 6a). CTOP and chelerythrine administered to PAG prior to morphine blocked the S890 NR1phosphorylation increase (Fig. 6a and b). Mice pre-treated with intra-PAG CTOP (80 ng) or chelerythrine (5 ng) administration were submitted to western blot analysis as previously described. No increase in phosphorylation on S890 NR1 could be detected in PAG from mice administered with these treatments with respect to basal value (Fig. 7a and b). NMDA NR1 content was not afflicted by CTOP or chelerythrine intra-PAG administration (Fig. 7a). Morphine dose dependently (0.01–7000 μg/kg) increased the phosphorylation of S890 NR1 in PAG samples; the maximum increase was reached at the 1 μg/kg dose administration to mice and further increase was not obtained at the higher doses (Fig. 8a and b). NR1 content was not afflicted by morphine administration (Fig. 8a). No increase of phosphorylation on serine 896 NR1 could be detected in PAG from mice administered with morphine (data not shown). Immunoblots were re-probed for β-tubulin which served as a loading control; no % significant density difference was revealed for this protein between lanes of the same immunoblotting. The band for S890 NR1 was lost when the proteins on the blot were dephosphorylated by exposure to lamba phosphatases (Fig. 9).

Histology

Histology confirmed that 96% mice used in the experiments had cannula placement within the PAG (Fig. 10).

Discussion

Role of opioid and glutamatergic receptors in the mouse PAG in the morphine-induced hyperalgesic response

In our experiments, exposure of PAG neurons to selective μOR antagonist CTOP at the higher dose completely reversed the morphine low dose-induced acute hyperalgesic response and this effect was dose-dependent. Low dose morphine-induced hyperalgesia appears to be μOR mediated at this supraspinal site even if the μOR subtype implicated in the hyperalgesic response is presently unknown (Cadet *et al.* 2003). PAG contributes to a different range of behaviors which are related to functional subdivisions in this area. The hyperalgesic response induced by morphine occurred as a result of activation of μOR receptor in dorsal lateral PAG, an area evocating flight reactions which usually follow an excitatory stimulus (Morgan *et al.* 1998). Since lesioning/blocking supraspinal regions reduces the hyperalgesia induced by formalin, mustard oil or spinal nerve injury, it further supports the participation of PAG in the execution and facilitation of spinal nociceptive reflexes (Wiertelak *et al.* 1994; Mansikka *et al.* 1996; Wei *et al.* 1998; Urban and Gebhart 1999). There is also considerable evidence that the hyperalgesia or allodynia associated with nerve injury or inflammation depends on activation of NMDA receptors. For example, the administration of NMDA receptor antagonists, such as MK801, ketamine or memantine strongly reduces hyperalgesia or allodynia in animals, including man (Carlton and Hargett 1995; Eisenberg *et al.* 1998; Wei and Pertovaara 1999; Ozyalcin *et al.* 2004; Gottrup *et al.* 2006). Evidence for NMDA receptor activation in rodent brain during the hyperalgesic response to low dose morphine systemic administration was recently demonstrated (Holtman and

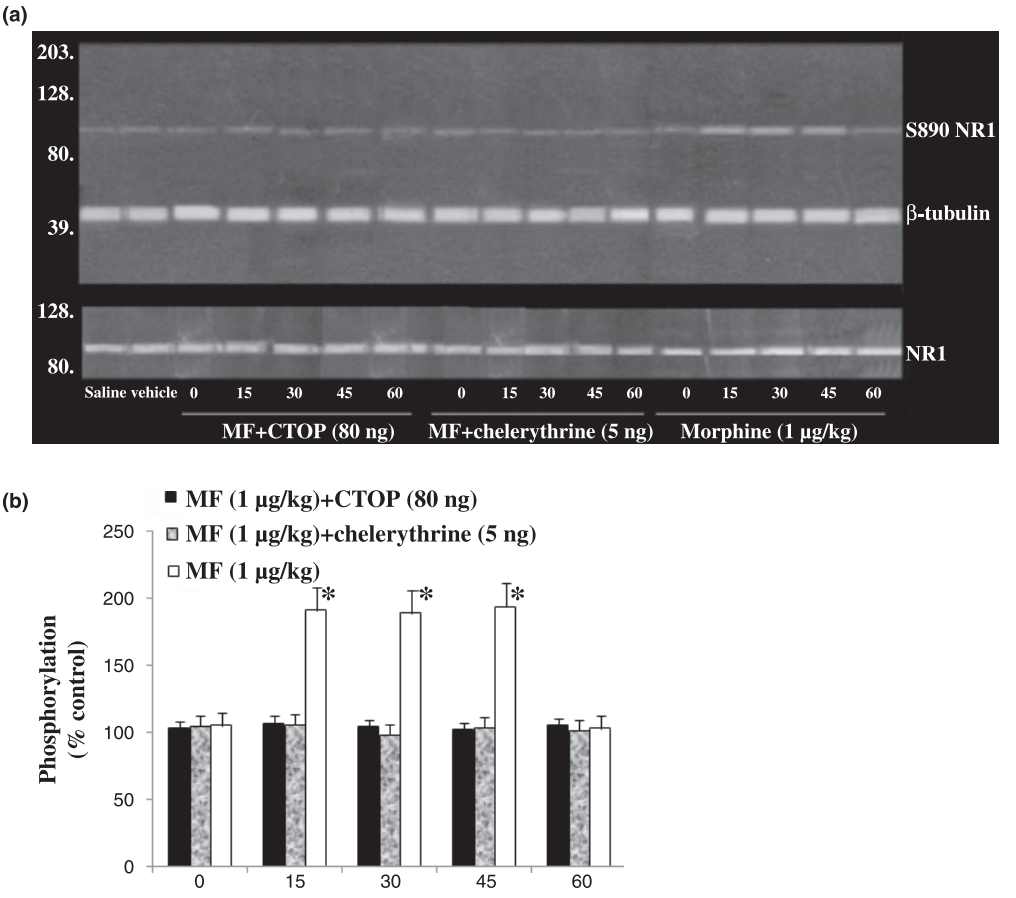

Fig. 6 Morphine administration (1 μg/kg, s.c.) significantly increases phosphorylation on NR1 S890 in PAG sample at 15, 30, and 45 min after morphine administration. Phosphorylation returns to basal level at 60 min after morphine administration. A representative immunoblot is shown with β-tubulin as loading control (a). Morphine (1 μg/kg, s.c.) administered in presence of selective μOR antagonist CTOP (80 ng, intra-PAG) or chelerythrine (5 ng, intra-PAG) failed to increase NR1 S890 phosphorylation (a). Anti-NR1 detection is reported in (a) using the membrane revealed for S890 phosphorylation previously stripped and re-incubated with anti-NR1 antibody. The mean + SEM of phosphorylation density values obtained in PAG from five different experiments are reported in (b). Values are expressed as percentage of density obtained in s.c. saline-treated mice; * indicates difference at $\alpha < 0.01$ significance level in comparison with control value. MF = morphine.

Wala 2005; Galeotti *et al.* 2006). When NMDA receptor in PAG was blocked by the selective antagonist MK801 at different doses, the activation of μOR by morphine low dose could not induce the hyperalgesic response in presence of the higher MK801 dose showing that opioid and glutamatergic receptors in PAG are both implicated in inducing the hyperalgesic response elicited by systemic morphine administration. At the same time, acute thermal hyperalgesic response induced by morphine low dose administration to mice is lacking in presence of the selective PKC inhibitor chelerythrine administered intra-PAG showing that the hyperalgesic effect is mediated by this kinase at PAG level. *In vitro*, μOR stimulation has been shown to start the activation of NMDA receptors by increasing intracellular PKC activity in trigeminal neurons (Chen and Huang 1991) as well as translocation of the cytosolic PKCγ to the plasma membrane leading to phosphorylation of the NMDA receptors implicated in pain promotion (Suen *et al.* 1998). It would appear that low dose morphine μOR coupling might initiate PKC translocation and activation which causes a removal of Mg^{++} blockade of NMDA receptor (Chen and Huang 1992). With this blockade removed, even small amounts of glutamate ligands, which could be basally released from pre-synaptic terminals, may activate the NMDA receptor and allow localized Ca^{++} channels to open triggering the excitatory response.

NMDA receptor phosphorylation in PAG neurons in morphine induced hyperalgesic response

NMDA receptors are phosphorylated and dephosphorylated by a variety of kinases that regulate NMDA receptor function. Different reports have shown that phosphorylation

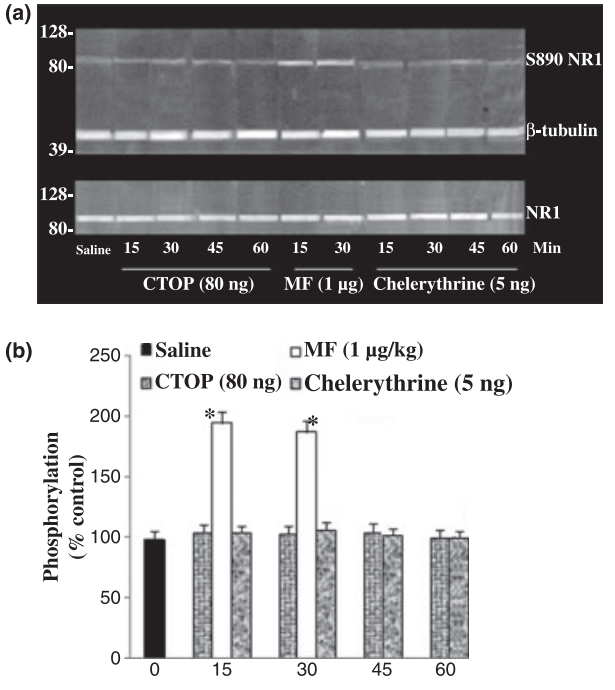

Fig. 7 CTOP (80 ng) and chelerythrine (5 ng) intra-PAG administration to mice does not increase NR1 S890 phosphorylation in PAG sample at 15, 30, 45 and 60 min after administration (a). Morphine s.c. administration (1 μg/kg), used as positive control, significantly increases phosphorylation on NR1 S890 at 15 and 30 min after administration. A representative immunoblot is shown with β-tubulin as loading control (a). Anti-NR1 detection is reported in (a) using the membrane revealed for S890 phosphorylation previously stripped and re-incubated with anti-NR1 antibody. The mean + SEM of phosphorylation density values obtained in PAG from five different experiments are reported in (b). Values are expressed as percentage of density obtained in saline-treated mice; * indicates difference at $\alpha < 0.01$ significance level in comparison with control value (0 time). MF = morphine.

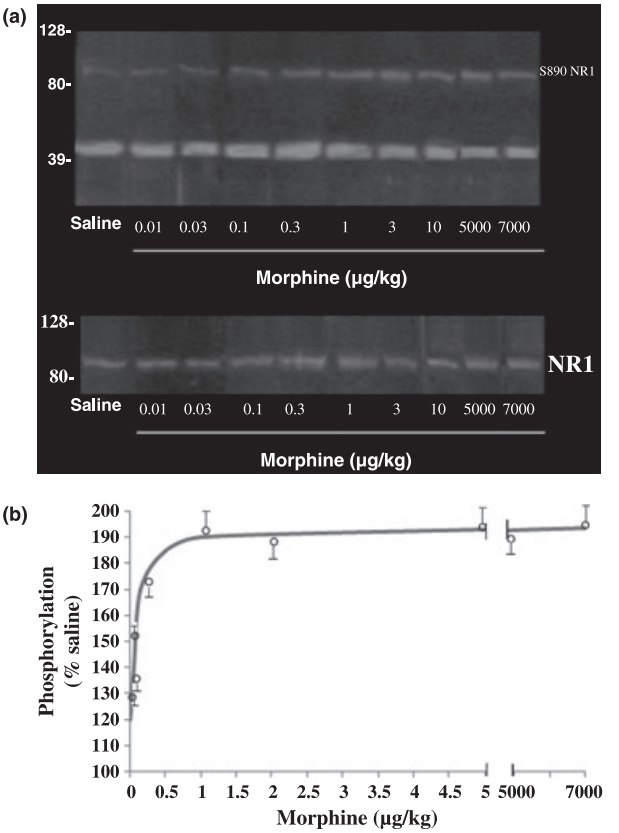

Fig. 8 Morphine administration at different doses (0.01–7000 μg/kg) dose dependently increase the phosphorylation of NR1 S890 in PAG sample (a). The higher density values were obtained at 1–7000 μg/kg dose administration to mice (a and b); * indicates difference at $\alpha < 0.01$ significance level in comparison with saline value. A representative immunoblotting is shown with β-tubulin as loading control (a). NR1 content is shown in the same membrane previously stripped and re-incubated with anti-NR1 antibody (a). The fitting of phosphorylation density with respect to morphine concentration to the mathematical eqn (1) is reported in (b). A value of 192 ± 12% and 0.08 ± 0.04 μg were obtained respectively for E_{max} and EC_{50} (b).

of the NMDA NR1 subunit is associated with noxious stimulation demonstrated by capsaicin, carrageenan and formalin injection to rodents (Zou *et al.* 2002; Caudle *et al.* 2005; Kim *et al.* 2006). Particularly, PKCγ effects the NR1 subunit phosphorylation at serine 890 as well as cyclic AMP-dependent PKCα mediates phosphorylation at serine 896 (Sanchez-Perez and Felipo 2005). In our experiments, the morphine-induced hyperalgesic effect is accompanied by an increase in phosphorylation of serine 890 aminoacidic residue in the NR1 subunit of the NMDA receptor in PAG whereas no phosphorylation increase could be detected on serine 896 in the same experimental conditions. NR1 subunit appeared to be higher phosphorylated on serine 890 with respect to basal value in PAG samples from mice killed at 15 and 30 min after morphine administration. At these times, a thermal hyperalgesic response was observable. Phosphorylation increase on NR1 subunit S890 was still observable at 45 min after morphine administration when the hyperalgesic response was reversed to basal level. A return to basal phosphorylation level could be observed at 60 min after morphine administration and, at that time, no hyperalgesic effect could be revealed. Our experiments show that the phosphorylation of subunit 1 of NMDA receptor and the acute thermal hyperalgesia are temporally related even if there is an inconstancy in the time course of phosphorylation of NR1 S890 by morphine and morphine-induced hyperalgesia. The later return to basal value of NMDA receptor phosphorylation induced by activation of kinases was previously observed. Phosphorylation increase of NR1 by PKC on serine 890 in hippocampus lasted for the first 3 h following the termination of drug induced status epilepticus in rat before returning to basal values (Niimura *et al.* 2005). Phoshorylation on serine 890 in mouse PAG appeared to be

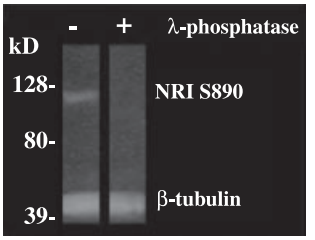

Fig. 9 Western blot of mice PAG sample revealed for NR1 S890 phosphorylation in the presence (right side) or the absence (left) of λ-posphatase. β-tubulin was used as loading control.

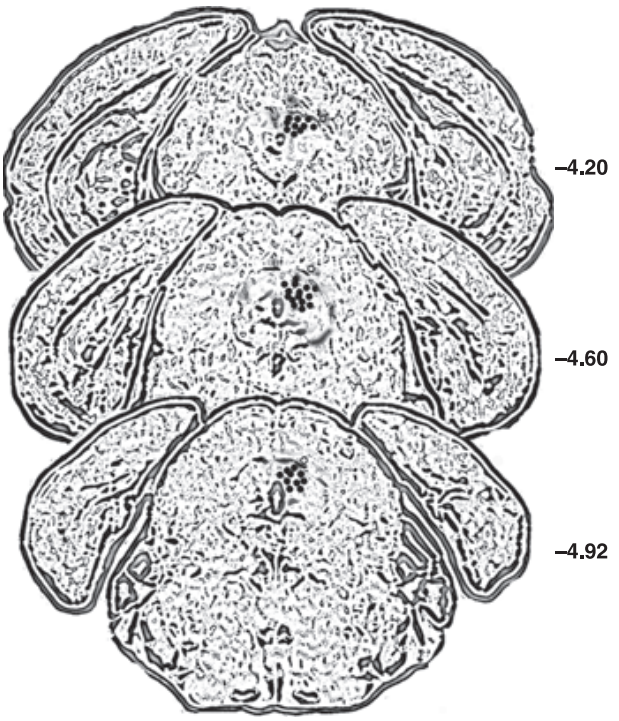

Fig. 10 Schematic representation of microinfusion site distribution within (filled circle) and outside (black circle) PAG area of brain mouse. The number of the filled circles is less than the total number of mice because of overlaps. The distance from bregma is represented on right side.

morphine dose-dependent and the maximum phosphorylation effect was obtained at 1 μg/kg dose administration to mice. Higher morphine doses failed to further increase phoshorylation level confirming that phosphorylation increase on serine 890 is saturated at hyperalgesic doses. The increase in phosphorylation of NR1 S890 in PAG induced by morphine systemic administration to mice was blocked in presence of a selective μOR antagonist injected directly to PAG demonstrating that NR1 receptor alteration is triggered by μOR ligand in this brain region. Morphological evidence showed that the majority of NR1 labeled dendrites contained μOR labeling in PAG (Commons *et al.* 1999). Co-immunoprecipitation studies revealed that NR1 is physically associated with functional active PKC; particularly, PKCγ is associated physically with NR1 subunit in the rat brain suggesting that these receptors may be directly phosphorylated by the kinase (Suen *et al.* 1998). In our experiments, the inhibition of PKC reverts to basal value the NR1 S890 phosphorylation increase in PAG induced by morphine hyperalgesic dose confirming that NR1 phosphorylation in this area is induced by PKC activation.

Conclusions

Taken the data as a whole, our findings show that a site of action of morphine low dose on acute thermal hyperalgesic response is located at the PAG. At this supraspinal site, μOR stimulation by systemically morphine low dose administration leads to an increased phosphorylation of specific subunit of NMDA receptor suggesting a role for the NR1 subunit of the NMDA receptor in morphine-induced hyperalgesia. These data strongly imply a molecular interaction, at supraspinal level, between NMDA and opioid receptors in morphine-induced acute nociception. PKC appears to be the key element that links μOR activation by morphine low dose with the recruitment of the NMDA/glutamatergic system in PAG. Our finding agrees with previous evidence of active pronociceptive processes initiated by opioid administration (Mao 2002). Preclinical studies have shown that systemic opioid administration in rodents can lead to an abnormal pain sensitivity as shown by a long-lasting reduction of baseline nociceptive threshold (Laulin *et al.* 1998); opioid-induced pain sensitivity can develop rapidly after an acute systemic opioid exposure as observed in human volunteers (Guignard *et al.* 2000; Angst *et al.* 2003). The central glutaminergic system plays a pivotal role in these neural mechanisms and NMDA receptor has been shown to be critical in the cellular mechanisms of opioid-induced pain sensitivity (Chen and Huang 1991; Mao *et al.* 1995). Hyperalgesia is observed both in animal model after administration of low morphine dose or as a delayed response seen subsequent to the analgesic response produced by central or systemic administration of opioid agonists to humans (Celerier *et al.* 2000; Mao 2002; Richebe *et al.* 2005). These paradoxical effects were usually ascribed to progressive reduction of opioid effects, occurring over a period of weeks, with repeated drug administration as in tolerance or following a single opiate administration as in short-term tolerance. Our findings provides some preclinical evidence that tolerance – especially short-term tolerance – may be not mainly due to a decrease in opioid effectiveness, but might result from the activation of nociceptive facilitatory systems at later times after morphine analgesic dose administration when the opioid concentration is expected to be as low as morphine concentration at supraspinal site after low dose administration.

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